Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 345 (2006) 220 – 230

VIROLOGY

www.elsevier.com/locate/yviro

Matching complementing functions of transformed cells with stable expression of selected viral genes for production of E1-deleted adenovirus vectors

John A. Howe *, Peter Pelka, Doug Antelman, Chris Wilson, Doug Cornell, Wendy Hancock, Murali Ramachandra, Jenny Avanzini, Mark Horn, Ken Wills, Suganto Sutjipto, Robert Ralston

Biological Research, Virology, Schering-Plough Research Institute, 2015 Galloping Hill Road, K-15-E403C, 4945, Kenilworth, NJ 07033, USA

Received 22 July 2005; returned to author for revision 23 August 2005; accepted 8 September 2005
Available online 24 October 2005

Abstract

Production of E1-deleted adenovirus (rAd) vectors requires complementation by E1A and E1B functions provided by the production cell line. The two cell lines most commonly used for production of rAd vectors, 293 and Per.C6, were derived from human primary cells and contain contiguous E1A and E1B sequences from the Ad genome. As an alternative system, we tested complementation of rAd vectors using sequential transfection of individual E1A and E1B expression cassettes into A549 human lung tumor cells, which support highly efficient replication of wild type adenovirus. We found that E1A function could be complemented in A549 cells by the mutant E1A*dl*01/07, and that E1B function could be provided in such cells using only the 55K E1B gene. Production yields in the resulting producer cell line, designated SL0003, were similar to those obtained from 293 cells without generation of detectable recombinant replication competent adenovirus.

Keywords: Adenovirus; Gene Therapy; Production cells; E1A; E1B

Introduction

Replication defective adenovirus (rAd) vectors have desirable features for gene therapy including wide tissue and cell tropism, the capacity to accommodate large expression cassettes and high transduction efficiency. In addition, adenovirus is well suited for pharmaceutical development as the virus grows to high specific titers and scalable manufacturing processes have been established (Huyghe et al., 1995; Shabram et al., 1997). Production of rAd vectors requires engineered cell lines that can complement functions removed from the viral genome. For pharmaceutical development, the vector-cell line combination also must be amenable to scale-up and provide material of sufficient quality and purity. In this work, we describe a new approach to production of rAd vectors based on combining selected viral and host functions required for adenovirus replication.

Replication defective rAd vectors for gene therapy use are generally deleted for the viral early region 1 (E1). E1 contains two transcription units, E1A and E1B that encode a number of proteins that have critical roles in the early and late phases of the lytic cycle and production of rAd-vectors requires complementation of these E1 activities. E1A and E1B gene functions have been extensively characterized [for review see (Bayley and Mymryk, 1994; Shenk, 1996)]. The first E1-complementing line, 293, was generated by transfection of primary embryonic kidney cells using physically sheared adenovirus 5 DNA (Graham et al., 1977). Genomic analysis subsequently demonstrated that 293 cells carry an integrated fragment of the left-hand end of adenovirus genome (bases 1–4344), containing the E1 region and additional flanking sequences (Louis et al., 1997).

Although 293 cells produce E1-deleted rAd vectors at acceptable levels, an undesirable contaminant called replication

* Corresponding author. Fax: +1 908 740 3032.
E-mail address: john.howe@spcorp.com (J.A. Howe).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.09.029

competent adenovirus (RCA) is sometimes generated by homologous recombination between the rAd vector and the adenovirus sequences present in the 293 genome (Lochmuller et al., 1994; Zhu et al., 1999). To reduce the risk of generation of RCA by homologous recombination, Fallaux et al., 1998 transfected human embryonic retinoblasts with a recombinant plasmid containing E1 genes, in which the E1A promoter and E1B polyadenylation sequences were replaced by heterologous control elements. Deletion of the adenovirus flanking sequences in the E1 plasmid yielded a cell line, PER.C6, which does not generate RCA through homologous recombination when cell line matched rAd-vectors are produced (Fallaux et al., 1998). However, recent studies have shown that an atypical form of RCA, called helper-dependent E1-positive particles, can be formed when non-matched adenoviral vectors are propagated in PER.C6 cells (Murakami et al., 2002).

Although both 293 and PER.C6 cell lines have proved useful tools for production of rAd vectors, consideration of recent work on oncolytic adenoviruses as well as the approaches taken in developing retroviral producer cells suggested an alternative approach to development of adenovirus production cell lines. Oncolytic adenoviruses have been developed for cancer therapy, based on the observation that human tumor cell lines can provide growth control and anti-apoptotic functions that are normally supplied by the E1A and E1B genes of adenovirus (Dobbelstein, 2004). Thus, adenoviruses with specific deletions affecting E1A or E1B can replicate efficiently in human tumor cell lines, but are defective for growth in primary cells. For example, dl01/07 virus contains two deletions in the E1A gene (see Fig. 1) that limit E1A-induced cell cycle progression and viral replication in normal cells (Doronin et al., 2000; Howe et al., 2000). However, dl01/07 can grow as efficiently as wild type virus in several human tumor lines including HeLa and A549, which have been widely used for culture of wild type adenovirus (Smith et al., 1986). In the retrovirus field, production cell lines have been developed in which each complementing function is supplied by an individual cassette expressing a single retrovirus gene, with each cassette introduced in a separate transfection step to further reduce the potential for homologous recombination during vector production (Palu et al., 2000).

In this paper, we describe a novel approach to complementation of E1 function by use of a human tumor cell line, A549, transformed with transgenes expressing selected E1A and E1B activities. The salient features of this approach include: sequential addition of E1A and E1B expression cassettes for stable expression of select E1A and E1B functions; use of the transformation defective E1A*dl*01/07 mutant to reduce E1A induced toxicity in A549 cells; definition of the E1B functions required for complementation with a genetic assay; and, complementation of E1B-55K without E1B-19K to provide E1B function. The cell lines resulting from this approach produce rAd-vectors efficiently without detectable RCA.

Results

Virus production potential in selected cell lines was assessed by infection with the replication competent virus Ad5-*dl*309. A549 cells and HeLa cells produced virus at 558,000 and 444,000 particles/cell, respectively. Other lines tested including, DLD-1, U87MG, MDA468 and IGROV-1, all produced less than 120,000 particles/cell (data not shown). 293 cells produced Ad5-*dl*309 virus at 139,000 particles/cell and rAd-β-gal virus at 127,000 particles/cells. The production capacity for Ad5-*dl*309 in HeLa and A549 suggested that these human tumor lines were the best candidates for use in development of E1 complementing cell lines.

Complementation of E1A in A549 cells

A549 cells were chosen over HeLa cells for further development because they do not contain papillomavirus sequences (Oh et al., 2004). Expression of wild-type E1A is

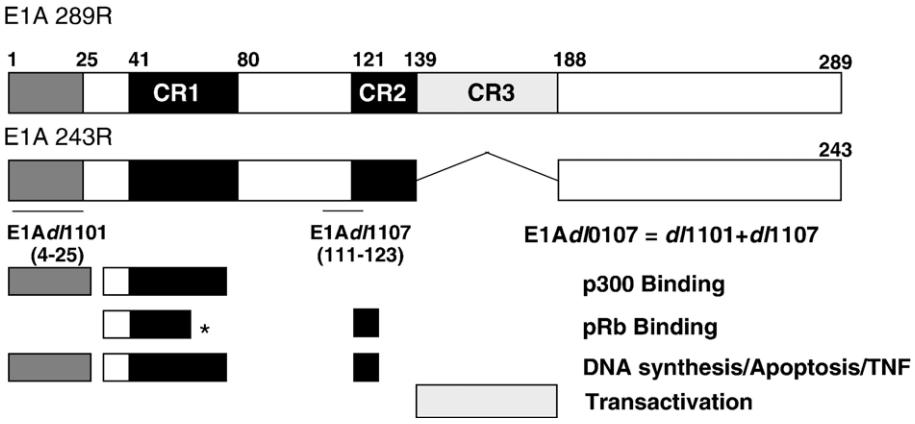

Fig. 1. E1A functional domains and mutants used in this study. Schematic representation of the 289R and 243R E1A proteins illustrating conserved regions 1 (CR1), CR2 and CR3, and domains involved in; binding of the cellular proteins p300/CBP and pRb, stimulation of cell cycle progression, induction of apoptosis and susceptibility to tumor necrosis factor (TNF), and transcriptional activation of the adenovirus early gene regions. Amino acid residues deleted in the wild-type E1A sequence by the E1A*dl*1101 and E1A*dl*1107 mutations are indicated.

known to reduce the proliferative capacity of A549 cells and leads to apoptosis under serum-depleted conditions (Hubbersteyet al., 2002). To limit the toxicities associated with E1A expression in A549 cells, we tested several E1A mutants for stable expression and complementation of E1 function for rAd-vector production. The E1A mutants used, E1A*dl*1101, E1A*dl*1107 and E1A*dl*01/07, carry deletions in regions of E1A proteins that are required for induction of cell cycle progression and apoptosis (Fig. 1) but do not compromise the E1A transactivation domain required for stimulation of the other early virus promoters. Specifically, the E1A*dl*1101 and E1A*dl*1107 mutants are defective for binding to the cellular proteins p300/CBP and pRb, respectively, whereas the E1A*dl*01/07 mutant is defective for binding both cellular proteins (Howe et al., 1990; Howe and Bayley, 1992).

Plasmids expressing either E1A*dl*1101, E1A*dl*1107, E1A*dl*01/07 or wild-type E1A under control of an RSV promoter were used to transfect A549 cells. The plasmids also contained a neomycin resistance gene as a selectable marker. Individual clones from pools of G418 resistant cells were isolated and screened by infection with rAd-GFP. We visually examined the level of GFP expression in 48 clones and then selected five clones for further characterization. The production capacity of each clone was determined by quantification of rAd-GFP particles produced per cell as described in Materials and methods. Results of the production assays (Fig. 2A) show that the clones expressing either E1A*wt*, or E1A*dl*1101, had low virus productivity. Clones isolated from the E1A*dl*1107 generally gave poor production yields, with the exception of clone E1A*dl*1107-1 which had a yield of over 20,000 particles per cell. In contrast, clones isolated from the E1A*dl*01/07 transfection pool had the highest production yields as four of the five clones assayed yielded particle concentrations between 20,000 and 30,000 per cell.

Measurement of E1A protein levels in E1A expressing A549 clones

E1A protein levels in the selected clones were determined by Western blot analysis using an E1A-specific monoclonal antibody (Harlow et al., 1985) that recognizes an epitope in the C-terminal region (Fig. 2B). This epitope is unaffected by the *dl*1101 and *dl*1107 mutations. Western analysis showed that expression levels of E1A proteins varied greatly between the clones. In addition, the amount of wild-type or mutant E1A expressed did not correlate with the production yields. All clones expressing wild-type E1A or the E1A*dl*1101 mutant were inefficient producers regardless of the amount of E1A protein expressed. Among the E1A*dl*1107 clones, only E1A*dl*1107-1 was an efficient producer despite expressing nearly undetectable levels of E1A protein. The E1A*dl*1107-1 clone was not further analyzed as it grew poorly, a characteristic undesirable in a production cell line. The E1A*dl*01/07 clones also displayed various levels of protein with the best producer clone E1A*dl*01/07-4 expressing lower levels as compared to E1A*dl*01/07-5, which had comparatively high levels.

Analysis of viral replication and apoptosis after infection

The E1A*wt*-2 and E1A*dl*01/07-5 cell clones, which expressed similar levels of E1A proteins, were selected to

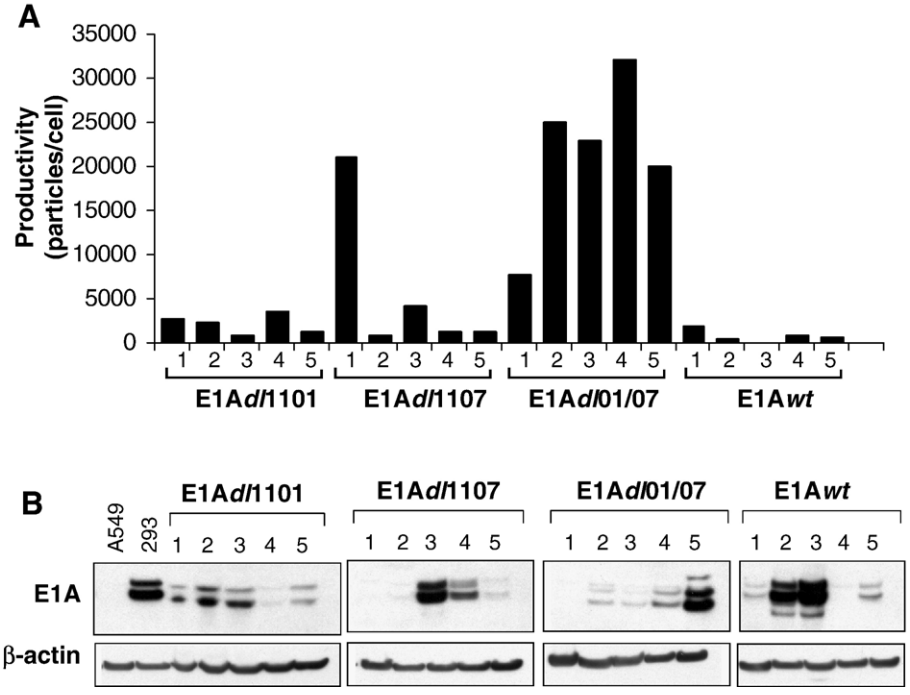

Fig. 2. Production capacity for rAd-vectors and E1A protein expression in the A549-based clones. (A) Virus yield from sets of 5 cell clones selected to express the indicated E1A mutants (E1A*dl*1101, E1A*dl*1107, E1A*dl*01/07) or wild-type E1A (E1Awt). (B) Relative E1A protein levels in lysates from the A549-based clones, expressing the indicated E1A mutants or wildtype E1A, were determined by Western blotting with antibodies specific for E1A or β-actin as a loading control.

further characterize the biological basis for the different production yields. Because the E1A*dl*01/07-4 clone produced viral particles more efficiently than any other line, it was also selected for further characterization.

The ability of the A549-E1A*wt*-2 and two A549-E1A*dl*01/07 clones to support replication of an E1-deleted virus rAd-β-gal virus was analyzed by restriction enzyme digest of low molecular weight DNA from infected cells. Restriction analysis indicated that there were differences in the quantity and quality of viral DNA isolated from the various cell lines (Fig. 3A). The quality of rAd-β-gal DNA from the E1A*dl*01/07 cells was comparable to 293 cell isolated viral DNA, although detected at somewhat reduced levels particularly in the A549-E1A*dl*01/07-5 clone. Extremely low levels of rAd-β-gal viral DNAs were produced from the E1A*wt*-2 clone, and the DNA that was isolated smeared on the agarose gel with DNA fragmentation suggesting high levels of apoptosis in these cells.

Analysis of viral DNA produced from clone E1A*wt*-2 suggested that apoptosis could be occurring, potentially limiting the amount of virus produced. We used a FITC-VAD-FMK peptide to measure caspase activity by flow cytometry as an indicator of apoptotic cell death after infection. As shown in Fig. 3B, almost half of the E1A*wt*-2cells were apoptotic 20 h after infection, whereas apoptosis was not induced in the E1A*dl*01/07-4 and E1A*dl*01/07-5 clones. Together, these results suggest that viral infection of the E1A*wt*-2 induces premature apoptosis, limiting viral DNA replication and subsequent virus particle production.

Status of the E2F and p53 transcription factors in E1Awt and E1Adl01/07 clones

Induction of apoptosis by E1A has been shown to be linked to binding of pRb and p300/CBP, resulting in activation of the E2F and p53 transcription factors. To measure E2F and p53 activity levels in the selected production cell clones, we constructed reporter plasmids in which a luciferase gene was placed under control of either a consensus p53 or E2F promoters. p53 and E2F promoter activity was found to be elevated in the E1A*wt*-2 clone (Fig. 3C). In contrast, p53 and E2F activity levels in the E1A*dl*01/07-4 and E1A*dl*01/07-5 clones were similar to the control A549 cells.

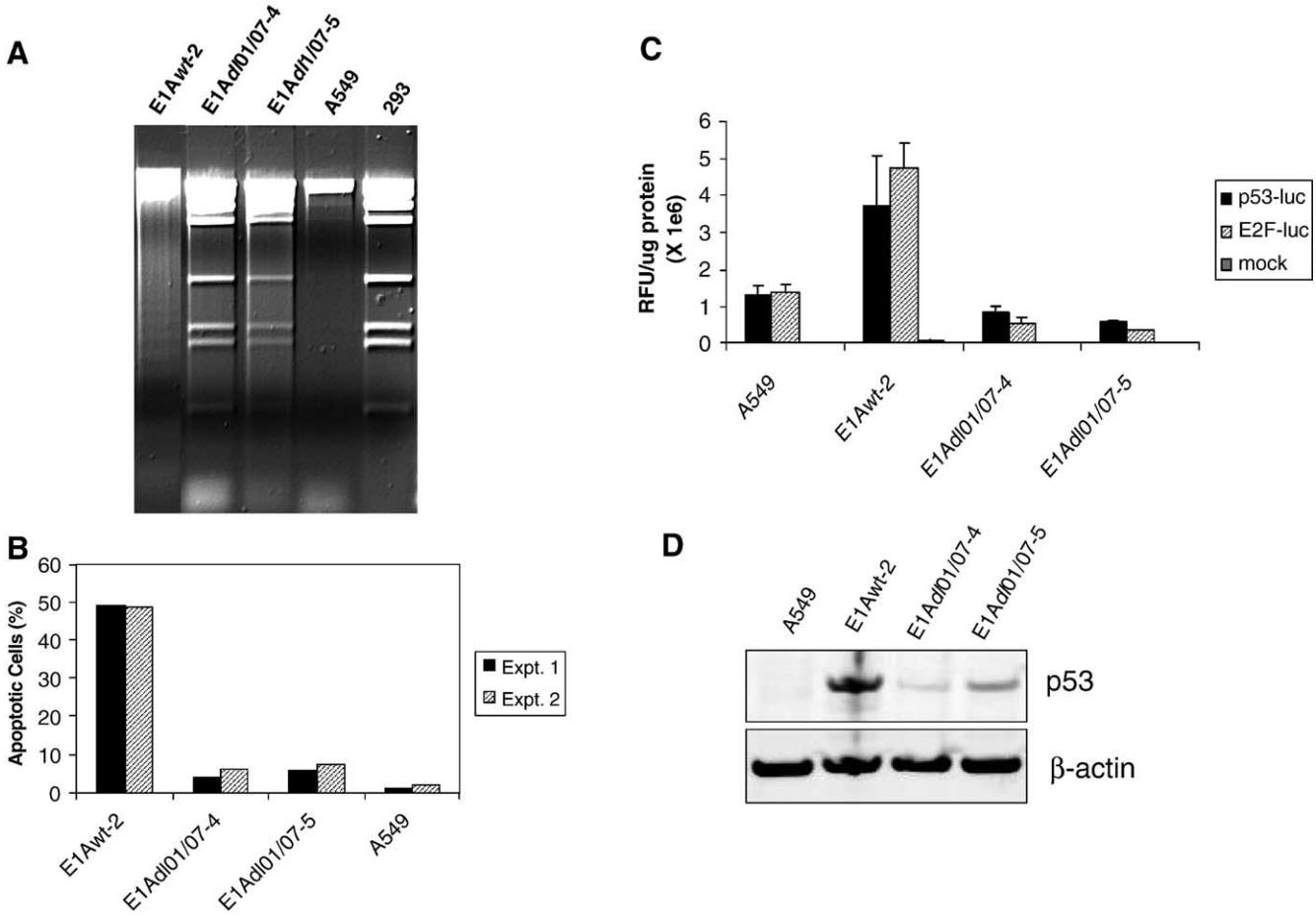

Fig. 3. Characterization of the E1A*wt*-2, E1A*dl*01/07-4 and E1A*dl*01/07-5 clones for viral DNA replication, induction of apoptosis after infection, E2F activity, p53 activity and protein level. (A) Low molecular weight DNA, isolated at 42 h after rAdvector infection of the indicated cell types, was digested with the *Xho*I restriction enzyme and analyzed by agarose gel electrophoresis. (B) Percentage of apoptotic cells as determined by FITC-VAD-FMK labeling at 20 h after rAd infection of the indicated cell types. (C) Luciferase activity measured in lysates of the indicated cell clones after transfection of reporter plasmids with the p53 (p53luc) or E2F (E2F-luc) responsive promoters linked to the luciferase coding sequence. (D) Relative p53 protein levels were measured in lysates of the indicated cell types, at 16 h after infection with rAd-p53 (1×10^8 particles/ml), by Western blotting with p53 specific monoclonal antibody and B-actin antibody as a loading control.

p53 protein levels in the selected clones were also determined by western blotting. A549 tumor cells express wild type p53 (Ramachandra et al., 2001). However, in preliminary experiments, significant levels of p53 protein could not be detected by Western blotting extracts of A549 cells or the selected A549 production cell clones. p53 has been shown to be highly unstable in A549 cells, likely as a result of loss of ARF expression, and the subsequent hyperactivation of the MDM2 feedback loop which promotes rapid p53 turnover (Lu et al., 2002). Therefore, we used an Ad vector, rAd-p53, as an exogenous source of p53 to measure the stability of p53 in A549 cells and the selected clones. After infection with rAd-p53, elevated levels of p53 were detected in the E1A*wt*-2 line compared to control A549 cells and the E1A01/07-4 and E1A*dl*01/07-5 lines (Fig. 3D).

Complementation of E1B in E1Adl01/07-4

Although the E1A*dl*01/07-4 clone was capable of producing replication defective adenovirus in small scale format, we reasoned that yield could be further improved by complementation using E1B. To study the production yield of E1A*dl*01/07-4 cells after E1B complementation E1A*dl*01/07-4 cells were infected, at two different concentrations (1×10^8 particles/ml and 5×10^8 particles/ml), with a series of E1 adenovirus mutants to express E1B-19K and E1B-55K either individually or together. We found that infection of E1A*dl*01/07-4 cells with a virus that produces a non-functional truncated E1A and wild-type E1B (Ad-NT1010) yielded virus comparable to Ad5-*dl*309 (Fig. 4). In contrast, a mutant virus that expressed E1A but no E1B (AdE1B$^-$) yielded only about 55,000 particles per cell after infection at both concentrations of test virus. These results suggested that complementation of E1B could significantly increase production yield. To further define the contribution of the E1B region, viruses expressing either E1B-19K (*dl*1520) or E1B-55K (AdE1B-19K$^-$) alone were tested for growth on E1A*dl*01/07-4 cells. The E1B-19K expressing virus productivity was considerably lower than Ad5-*dl*309 whereas the E1B-55K expressing virus produced at levels higher than Ad5-*dl*309.

These results suggested that addition of an E1B-55K gene could increase the viral yield in the E1A*dl*01/07-4 cell line. Use of only the E1B-55K coding sequences would further reduce the possibility of generating RCA during rAd production. We therefore used a selection plasmid in which the E1B-55K gene was cloned under control of the CMV promoter (pcDNA-55K) to complement E1B-55K function in E1A*dl*01/07-4 cells. The sequence of the expression cassette in pcDNA-55K was verified by DNA sequencing and expression of intact E1B-55K protein was demonstrated by transient transfection in HeLa cells (data not shown).

E1A*dl*01/07-4 cells were transfected with pcDNA-55K, which also carried a hygromycin resistance marker, and individual clones were selected from the drug-resistant pool by dilution cloning. Clones were screened for E1B-55K function using an assay that measured inhibition of a p53 responsive promoter (PRE) controlling GFP expression carried in a replication defective adenovirus named rAd-PRE-GFP. In cells expressing wild type p53, such as the parental E1A*dl*01/07-4 line, GFP is expressed at high levels from rAd-PRE-GFP. (Fig. 5A). In contrast, expression of GFP from rAd-PRE-GFP is low in cells such as 293, which also express E1B-55K and wild-type p53. Several clones including E1A*dl*01/07-4-E1B-55K-2 were selected using this assay and further characterized for E1B-55K expression and virus production.

Similar levels of E1B-55K protein were detected by immunoprecipitation, with the E1B-55K specific monoclonal antibody 2A6, in cell lysates from all of the selected clones (Fig. 5B) and one clone E1A*dl*01/07-4-E1B-55K-2, hereafter designed SL0003, was selected for further characterization based on its growth properties and virus production.

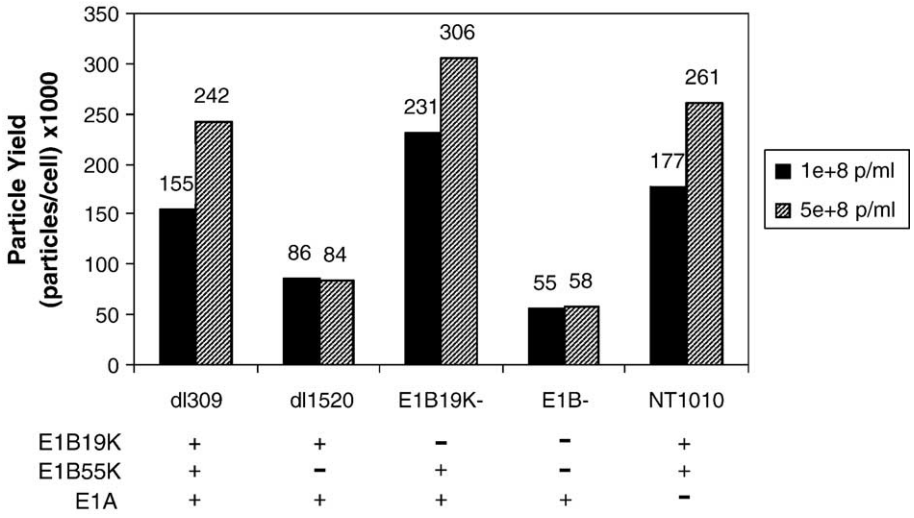

Fig. 4. Virus productivity after infection of E1A*dl*01/074 cells. Virus yield was measured from E1A*dl*01/07-4 cells infected with Ad5-*dl*309, *dl*1520, E1B-19K$^-$, E1B$^-$ or NT1010 adenoviruses at two different concentrations (1e+8 particles/ml and 5e+8 particles/ml). The expression status of E1B-19K, E1B-55K and E1A for each adenovirus is indicated.

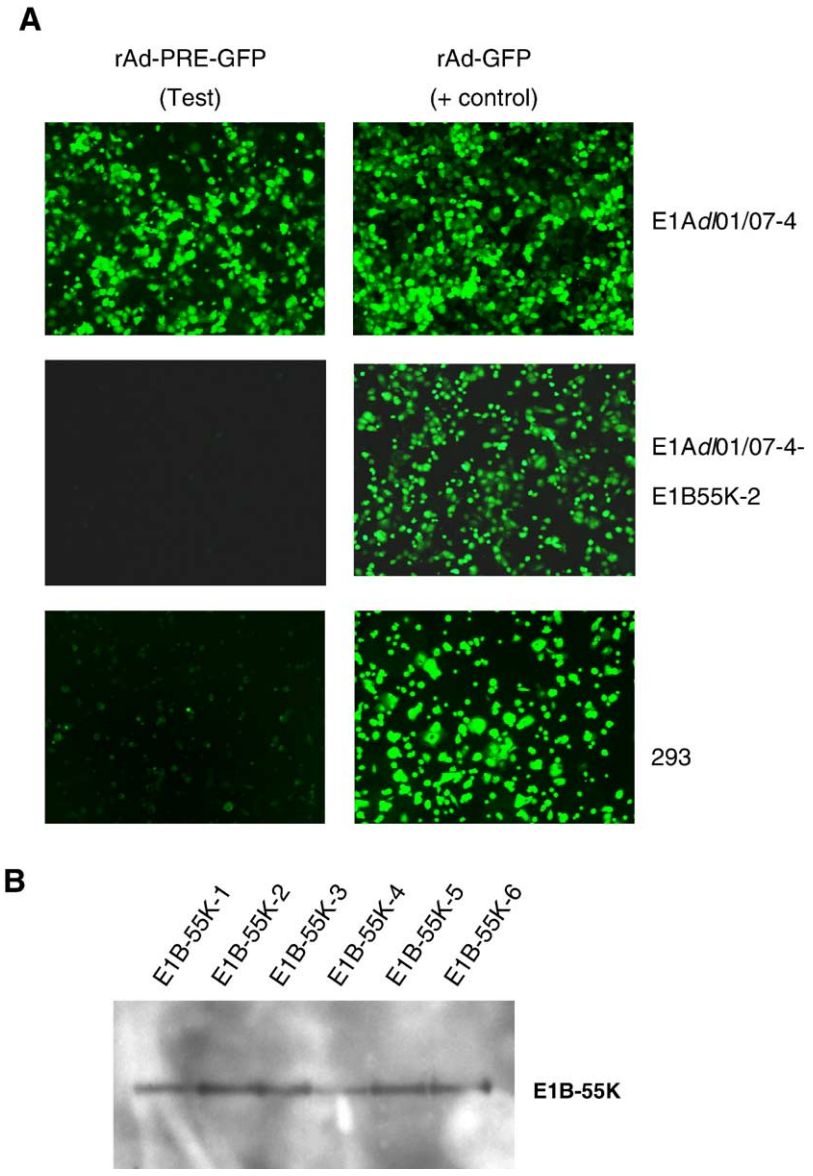

Fig. 5. Functional screening assay for E1B-55K expression in E1A*dl*01/07-4 cells. (A) E1A*dl*01/07-4, E1Adl01/07-4-E1B-55K-2 and 293 cells were infected with rAd-PRE/GFP, which contains a GFP expression cassette driven by a p53-responsive promoter, or rAd-GFP in which GFP expression is driven by the CMV promoter. (B) E1B-55K detected by immunoprecipitation/Western blotting of lysates from 6 selected E1A*dl*01/07-4 -E1B-55K clones.

Cell line stability is essential for pharmaceutical scale-up production and therefore the virus growth capacity of SL0003 cells was evaluated during 20 cell culture passages. Table 1 shows that SL0003 cells assayed at passage 5, 15 and 20 produced rAd-β-gal virus at an average of about 96,000 particles per cell.

RCA testing of virus produced in SL0003 cells

One of the goals in developing SL0003 cells was to reduce the possibility of RCA generation during production of Ad-vectors. To test for RCA generation during production, rAd-β-gal was serially passaged in either SL0003 or 293 cells grown in cell factories containing approximately 1×10^9 cells, using a RCA-free virus lot as the initial inoculum (see Materials and methods). The bioassay used for detection of RCA had a sensitivity of 1 particle of wild-type virus per 1.7×10^{10} particles of rAd-β-gal (see Materials and methods). Wild type Ad5 virus was used as a positive control in these assays. RCA was measured using 1×10^{11} particles of rAd-β-gal virus produced from either SL0003 or 293 cells. After 5 serial passages, RCA was detected in rAd-β-gal virus purified from 293 cells, but no RCA was detected in rAd-β-gal purified from SL0003. PCR analysis confirmed that the

Table 1
Virus yield from SL0003 cells

SL0003 passage no.	Particle no. (±SD)
5	88,800 ± 2800
15	105,600 ± 3700
20	93,000 ± 2800

virus detected in the RCA assay arose from recombination rather than contamination by Ad5 (data not shown).

Discussion

We have established a new rAd production cell line, SL0003, using selected viral and cellular functions to complement Ad replication in A549 tumor cells. Optimization of E1 complementation in SL0003 was achieved using sequential addition of separate E1A and E1B expression cassettes. In SL0003, the E1A*dl*01/07 mutant gene was constitutively expressed to provide E1A function and to reduce cellular toxicity associated with wild-type E1A. E1B function in SL0003 was provided by constitutive expression of the E1B-55K gene; the E1B-19K gene was not included. Separation of the E1A and E1B cassettes, use of a mutant E1A and only E1B-55K eliminates the possibility of reconstitution of an intact E1 region through either homologous or non-homologous recombination and resultant generation of RCA. Although the Ad sequences used for complementation in SL0003 are extensively modified compared to the wild-type E1 sequence in 293 cells, SL0003 was shown to produce rAd vectors at levels comparable to 293 without generation of detectable RCA.

SL0003 cells were established from a human tumor line, whereas the 293 and PER.C6 producer cell lines were established by adenovirus E1A-induced immortalization of primary human cells. Constitutive expression of the E1 proteins, especially E1A, has proven difficult in established cell lines (Imler et al., 1996). For example, E1A has been shown to suppress cell growth and induce anoikis (Frisch, 1991; Frisch and Mymryk, 2002; Mymryk et al., 1994; Rao et al., 1992). Imler et al. used regulated induction of E1 as a strategy to avoid toxicity associated with constitutive expression of E1A, allowing generation of rAd producer cell lines based on A549 cells (Imler et al., 1996). Production yields of rAd-vectors from these Gal4-inducible producer cell lines, which expressed E1A and E1B-19K but not E1B-55K, were reported to be 5- to 10-fold lower than from 293 cells (Imler et al., 1996).

An alternative approach to overcome the toxicity associated with wild-type E1A in tumor cells was to use mutant E1A genes to provide only the required functions. Previous work by our group and others has demonstrated that adenoviruses carrying the E1A*dl*01/07 mutation, or similar mutations affecting induction of cellular DNA synthesis and apoptosis, can replicate efficiently in human tumor cell lines but are attenuated for viral growth in normal human cells (Balague et al., 2001; Doronin et al., 2000; Howe et al., 2000). These results demonstrate that tumor cells, in which cell cycle control is aberrant, can complement the ability of mutated E1A genes to induce cellular DNA synthesis, a function required for efficient adenoviral growth in quiescent normal cells. In the present study, we took advantage of the unique properties of E1A*dl*01/07, which has mutations affecting cellular DNA synthesis and apoptosis but still carries the wild-type CR3 domain which transactivates early viral promoters required for the initial phase of lytic growth (Winberg and Shenk, 1984). Consistent with the growth properties of the E1A mutant viruses noted above, A549 clones that constitutively expressed the E1A*dl*01/07 gene supported replication of E1-deleted rAd vectors at levels higher than clones expressing wild-type E1A or the single mutations E1A*dl*1101 or E1A*dl*1107. In agreement with previous observations on E1A toxicity, A549 clones expressing wild-type E1A were especially sensitive to apoptosis following infection with E1-deleted rAd vectors and viral yields were extremely low.

E1A gene transfer has been reported to increase the sensitivity of cells to toxicity induced by TNF-α (Shisler et al., 1996) and chemotherapeutics (Ueno et al., 2000; Zhou et al., 2001), suggesting that E1A induces a pro-apoptotic state that can render cells highly sensitive to death stimuli (Lee et al., 2003). Increased TNF-α susceptibility was observed in NIH 3T3 cells expressing wild-type E1A or E1A mutants that could stimulate cellular DNA synthesis, but not in cells expressing E1A*dl*01/07 or other mutants that did not induce cellular DNA synthesis and consequently apoptosis (Shisler et al., 1996). The rapid induction of apoptosis in clone A549E1A*wt*-2 suggests that early events in viral infection may supply death signals that can be recognized by A549 cells expressing wild-type E1A but not E1A*dl*01/07. Recent studies have shown that retroviral expression of E1A can establish a pro-apoptotic state in cells through stimulation of the E2F pathway, leading to accumulation of caspase proenzymes and an increased susceptibility to cell death signals that trigger caspase activation and apoptosis (Nahle et al., 2002).

Apoptosis in adenovirus-infected cells can be stimulated though p53-dependent and p53-independent pathways (Teodoro et al., 1995). We found that both E2F and p53 pathways were stimulated in the A549*wt*-2 clone as compared to the A549E1A*dl*01/07 clones in which E2F and p53 transcriptional activity was similar to the parental A549 cells. The stimulation of the E2F and p53 pathways by wild-type E1A was not sufficient to induce apoptosis in the A549*wt*-2 clone, but likely contributed to the increased sensitivity of these cells to death stimuli. Consistent with this hypothesis, we found that A549E1A*wt*-2 cells could be driven into apoptosis by low levels of cycloheximide, but that A549E1A*dl*01/07 clones were unaffected by this treatment (data not shown). Further work will be required to define the apoptotic signals associated with adenovirus infection. Possible mechanisms include virus entry and internalization, which has been shown to activate the PI3K and MAPK pathways (Meier and Greber, 2003), viral DNA replication, or expression of viral genes such as E4orf4 (Marcellus et al., 1996).

The E1 region used for complementation of E1-deleted adenoviruses in 293 cells and PERC.6 cells includes the entire E1B transcription unit, which encodes two major proteins: E1B-19K and E1B-55K. In adenovirus replication, the E1B-19K and E1B-55K proteins function in the early lytic cycle to limit E1A-induced apoptosis (Querido et al., 1997; Rao et al., 1992; White et al., 1991). In addition, E1B-55K functions in the late phase to stimulate the accumulation and translational of viral late mRNAs (Babiss et al., 1985; Harada and Berk, 1999). E1B has also has been shown to collaborate with E1A in transforming primary cells [for review, see (Branton et al., 1985)], and specifically protects against E1A sensitization to apoptosis (White et al.,

1991). As described above, the mutant E1A*dl*01/07 was selected because it reduced sensitivity to apoptosis during vector production. We next sought to determine whether both E1B genes were needed for rAd vector complementation in the E1A*dl*01/07-expressing A549 cell line. Analysis of virus yield using a series of E1B-mutant viruses showed that in A549E1A*dl*01/07-4 cells, expression of E1B-55K alone was sufficient for production of the mutant viruses at wild-type levels. These results suggested that efficient complementation could be achieved in A549 cells by E1A*dl*01/07 plus E1B55K, which was subsequently demonstrated in the SL0003 line. The yield of rAd vectors from the SL0003 line was similar to that obtained from 293 cells, without generation of detectable RCA.

In conclusion, this study demonstrates that efficient production of E1-deleted rAd vectors can be achieved by matching complementing functions of transformed cells with stable expression of selected viral genes.

Materials and methods

Cell culture

293 (ATCC #CRL-1573), A549 (ATCC #CCL-185) and HeLa (ATCC #CCL-2) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (JRH Biosciences, Lenexa, KS), 1% (vol/vol) antibiotic–antimycotic solution (Cellgro, Kansas, MO) and 1 mM sodium pyruvate (BioWhittaker, Inc., Walkersville, MD).

Plasmids

The Ad5 wild-type E1A gene, and E1A sequences containing the E1A*dl*1101, E1A*dl*1107 and E1A*dl*01/07 mutations were cloned by standard procedures from pXC1 (McKinnon et al., 1982) or Ad-*dl*01/07 (Howe et al., 1990) into the RSV promoter/SV40 polyA expression cassette of pRc/RSV-Neo (Invitrogen, Carlsbad CA) to create pRc/RSV-E1A*wt*, pRc/RSV-E1A*dl*1101, pRc/RSV-E1A*dl*1107 and pRc/RSV-E1A*dl*01/07. pcDNA3.1-E1B-55K, was constructed by cloning the E1B-55K gene from pXC1 using PCR into the CMV promoter/BGH poly A expression cassette of pcDNA3.1-hygro (Invitrogen Carlsbad, CA). p53con-luc contains 4 consensus p53 binding sites, and a TATA box from the simian virus-40 (SV40) early promoter (Ramachandra et al., 2001), upstream of the luciferase gene in pGL3-basic (Promega, Madison, WI). pE2F-luc contains 4 copies of the E2F binding sites from the adenovirus early region 2 promoter and an SV40 TATA box upstream of the luciferase gene in pGL3-basic.

Viral constructs

rAd-β-gal and rAd-GFP are E1/E3 deleted adenovirus vectors with expression cassettes inserted in the E1-deletion in which the β-galactosidase (β-gal) gene, or green fluorescent protein (GFP) gene, are under control of the constitutively active CMV immediate early promoter (Cheney et al., 1998; Wills et al., 1994). The p53 reporter rAd-PRE-GFP contains an expression cassette in the E3-deletion in which a p53-response element (Ramachandra et al., 2001) regulates expression of GFP. Ad5-*dl*309 (Jones and Shenk, 1979) was used as a wild-type control virus, and the Ad5-*dl*309 based mutant viruses E1B/19K$^-$, which does not produce the E1B-19K protein (Marcellus et al., 1996), and NT1010, which has a large deletion in the E1A region (Whyte et al., 1989), have been described previously. *dl*1520 (Barker and Berk, 1987) is a chimeric adenovirus (Ad2 and Ad5-*dl*309) containing a deletion in the E1B coding region (Ad5 coordinates 2496–3233) and a stop codon at the third codon of E1B-55K. The Ad-E1B$^-$ virus was constructed by removing the E1B coding region in plasmid pXC1 by *Eco*NI and *Bgl*II digestion, Klenow fill in and blunt end ligation to create pXC1-E1B$^-$. Ad sequence containing the mutated E1B region was transferred from pXC1-E1B$^-$ into a larger transfer plasmid pTG9530 (Transgene S.A., Strasbourg), to create pTG9530-E1B$^-$. Homologous recombination in *E. coli* strain BJ5183 (Chartier et al., 1996) was used to generate infectious Ad-E1B$^-$ adenoviral DNA by transformation of pTG9530-E1B$^-$ and viral plasmid pTG4213-Ad5-*dl*309. The resulting Ad-E1B$^-$ plasmid was isolated and transfected into 293 cells to generate virus.

All viruses were purified by column chromatography using a method described previously (Huyghe et al., 1995). Particle concentrations were estimated by an ion exchange HPLC-based method (RQ-HPLC) that determines concentrations of intact adenovirus particles relative to an internal adenovirus standard (Shabram et al., 1997).

Selection of A549 cell clones expressing E1Awt, E1A-mutant proteins and E1B-55K

For selection of clones expressing E1A*wt* or E1A-mutant proteins plasmids pRc/RSV-E1A*wt*, pRc/RSV-E1A1101, pRc/RSV-E1A1107 and pRc/RSV-E1A01/07 were transfected into A549 cells using Superfect reagent (Qiagen, Valencia CA). After incubation for two days selection was initiated in growth medium containing 350 μg/ml neomycin (Invitrogen, Carlsbad, CA). Drug-resistant colonies from the cultures transfected with the E1A*wt*, or E1A-mutant selection plasmids, were trypsinized, established as cell pools, and dilution cloned in 96-well plates. Selection in culture medium containing neomycin was carried out 3 more weeks, after which 48 individual clones from each transfection were expanded and screened for virus production potential.

A549-E1A*dl*01/07-4 based cell lines were engineered to express E1B-55K using the same procedure except that A549-E1A*dl*01/07-4 cells were transfected with pcDNA3.1-E1B-55K and selected in 350 μg/ml hygromycin (Invivogen, San Diego, CA).

Screening procedures for cells clones

The virus production potential of A549-based clones expressing E1A*wt*, or E1A-mutant proteins, was assessed by infecting cells that were plated on 6-well plates with rAd-

GFP (1×10^8 particles/ml). Virus replication efficiency was estimated by monitoring green fluorescence protein intensity and cytopathic effect (CPE). For screening E1A01/07-4 based clones established by transfection with pcDNA3.1-E1B-55K cells were infected with rAd-PRE-GFP (1×10^8 particles/ml) and clones with low GFP intensity, suggesting reduced p53 activity, and robust CPE were chosen for further characterization.

Virus production in the selected clones was determined by measuring the number of particles produced on a per cell basis. For this assay, cells were infected with rAd vector at 5×10^8 particles/ml, and at the time of infection cells in duplicate plates or flasks were trypsinized and counted using a Coulter Counter (Beckman-Coulter, Miami, FL). At a time point when the infection was complete (3–4 days) cells and media were collected, freeze/thawed 3 times and centrifuged to remove cellular debris. The particle concentration in the cleared lysates was determined using anion-exchange high-performance liquid chromatography (Shabram et al., 1997).

Western blotting analysis to determine E1A protein levels

Whole cell protein lysates were prepared by incubation of the indicated cells in lysis A buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.5% [vol/vol] IGEPAL CA-630 [Sigma, St. Louis, MO] and protease inhibitor cocktail [Roche, Indianapolis, IN]) followed by centrifugation. Total protein concentration in the lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 50 μg aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with gradient (4%–12%) NUPAGE gels (Invitrogen, Carlsbad, CA). After transfer onto PVDF membranes (Invitrogen, Carlsbad, CA), western detection was performed using antibodies specific for E1A (M73, Calbiochem, La Jolla, CA) or B-actin (Sigma-Aldrich, St. Louis, MO). Binding patterns were determined by incubating the membranes with horseradish peroxidase-conjugated anti-mouse immunoglobulin G and M (Roche, Indianapolis, IN) and detected by enhanced chemiluminescence (Amersham BioSciences, Piscataway, NJ).

Analysis of viral DNA

Small molecular weight DNA was isolated from cells infected with rAd-β-gal (1×10^8 particles/ml) on 6-well plates at the indicated time points using a modified Hirt extraction (Hirt, 1967). Infected cells were harvested by scraping and lysed in TNE buffer [500 mM NaCl, 10 mM Tris (7.5), 10 mM EDTA, 1% SDS, 0.5 mg/ml proteinase K (Sigma), 0.25 mg/ml, pronase E (Sigma)]. After a freeze–thaw cycle lysates were cleared by centrifugation, extracted with a phenol:chloroform mixture (Sigma, St. Louis, MI) and ethanol precipitated. The nucleic acid pellets were suspended in 60 μl of TE containing RNase (Ambion, Austin, TX), after which 12 μl samples were restricted with *Xho*I, separated on 1% agarose gels. DNA restriction patterns were visualized by ethidium bromide staining.

Flow cytometric determination of apoptosis with FITC-VAD-FMK

E1A*wt*, E1A-mutant clones and A549 control cells were plated on 6-well plates at 1.5×10^5 cells per well and infected at 24 h with rAd-β-gal (1×10^8 particles/ml). At 20 h after infection, 5 μM CaspACE FITCTM-VAD-FMK (Promega, Madison, WI) was added directly to the culture medium. After incubation for 20 min at 20 °C the cells were trypsinized, washed 2 times with PBS and fixed in 0.5% formaldehyde for 30 min at 20 °C. Flow cytometry analysis was performed using an FACSCalibur (Becton Dickinson, San Jose, CA) and fluorescence was measured at 530 nm (excitation of 488 nm).

Luciferase assays

Lysates of cells transfected with reporter plasmids using Superfect (Qiagen, Valencia, CA), were mixed with the reconstituted luciferase substrate (Promega, Madison, WI) according to the manufacturer's specifications. Luciferase activity of each lysate was determined using an Analyst AD (Molecular Devices, Sunnyvale, CA).

Immunoprecipitation of E1B-55K

Protein lysates (1 mg total protein) prepared as described above were pre-cleared with protein-G sepharose beads (Amersham BioSciences, Piscataway, NJ) and incubated with 5 μg of the E1B-55K specific mouse monoclonal antibody, 2A6 (Sarnow et al., 1982). E1B-55K–immunoglobulin complexes were purified on protein-G sepharose, washed extensively with lysis buffer, and incubated with SDS-PAGE sample buffer containing reducing agent (Invitrogen, Carlsbad, CA). The samples were separated on SDS-PAGE gels and E1B-55K protein was detected by Western blot as described above, using the 2A6 monoclonal antibody.

Serial passage of an E1-deleted rAd-vector in 293 or SL0003

The rAd-β-gal virus used for serial passage was plaque purified three times, propagated using SL0003 cells grown in a cell factory (Nunc A/S, Kamstrupvej, Denmark) and after chromatographic purification the resulting virus stock, called rAd-β-gal (p0), tested free of replication competent adenovirus (RCA) using the 21-day bioassay described below. For serial passage infection, cell factories containing 293 or SL0003 were infected with purified rAd-β-gal (p0) at 5×10^8 particles/ml. Cell lysates were prepared when the infections were complete and were used to infect fresh 293 or SL0003 cells seeded in cell factories to prepare the passage 1 lysate. Lysates for infection of passages 2 to 5 were prepared similarly, and at passage 5, virus was purified by column chromatography as described above.

Assay for RCA

A modified bioassay based on a previously described protocol (Zhu et al., 1999) was used to detect RCA using 1 ×

10^{11} total particles of rAd-β-gal purified after propagation in 293 or SL0003. For the initial RCA bioassay infection 10-T225 flasks were seeded with 1×10^7 cells of A549 cells. At 24 h after seeding, the cells were infected with 1×10^{10} particles per flask of rAd-β-gal which was prepared from 293 or SL0003 cells. After 3 days, a cell lysate was prepared from the infected A549 cells and used to infect a second set of flasks that had been seeded with 3×10^6 A549 cells. For the second infection, half of the rAd-β-gal lysates from the first infections were used to infect 5-T225 flasks. At 12 days after, the initial infection lysates from the second round of infections were prepared and used to infect 10-flasks seeded with 3×10^6 A549 cells. The third infection procedure was preformed as for the second infection and incubated for 9 more days. If CPE was observed during the bioassay, low molecular weight DNA was isolated from approximately 1×10^7 cells for viral DNA analysis. Controls included 1×10^{11} virus particles of rAd-β-gal spiked with 6 virus particles of Ad5 wild-type, or 6 virus particles of Ad5 wild-type alone using the infection procedure described above. Both controls were required to produce CPE during the 21-day infection window for a positive assay.

References

Babiss, L.E., Ginsberg, H.S., Darnell Jr., J.E., 1985. Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. Mol. Cell. Biol. 5, 2552–2558.

Balague, C., Noya, F., Alemany, R., Chow, L.T., Curiel, D.T., 2001. Human papillomavirus E6E7-mediated adenovirus cell killing: selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes. J. Virol. 75, 7602–7611.

Barker, D.D., Berk, A.J., 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology 156, 107–121.

Bayley, S.T., Mymryk, J.S., 1994. Adenovirus E1A proteins and transformation (Review). Int. J. Oncol. 5, 425–444.

Branton, P.E., Bayley, S.T., Graham, F.L., 1985. Transformation by human adenoviruses. Biochim. Biophys. Acta 780, 67–94.

Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., Mehtali, M., 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J. Virol. 70, 4805–4810.

Cheney, I.W., Johnson, D.E., Vaillancourt, M.T., Avanzini, J., Morimoto, A., Demers, G.W., Wills, K.N., Shabram, P.W., Bolen, J.B., Tavtigian, S.V., Bookstein, R., 1998. Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated MMAC1/PTEN gene transfer. Cancer Res. 58, 2331–2334.

Dobbelstein, M., 2004. Replicating adenoviruses in cancer therapy. Curr. Top. Microbiol. Immunol. 273, 291–334.

Doronin, K., Toth, K., Kuppuswamy, M., Ward, P., Tollefson, A.E., Wold, W.S., 2000. Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. J. Virol. 74, 6147–6155.

Fallaux, F.J., Bout, A., van der Velde, I., van den Wollenberg, D.J., Hehir, K.M., Keegan, J., Auger, C., Cramer, S.J., van Ormondt, H., van der Eb, A.J., Valerio, D., Hoeben, R.C., 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum. Gene Ther. 9, 1909–1917.

Frisch, S.M., 1991. Antioncogenic effect of adenovirus E1A in human tumor cells. Proc. Natl. Acad. Sci. U.S.A. 88, 9077–9081.

Frisch, S.M., Mymryk, J.S., 2002. Adenovirus-5 E1A: paradox and paradigm antioncogenic effect of adenovirus E1A in human tumor cells. Nat. Rev., Mol. Cell Biol. 3, 441–452.

Graham, F.L., Smiley, J., Russell, W.C., Nairn, R., 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59–74.

Harada, J.N., Berk, A.J., 1999. p53-Independent and -dependent requirements for E1B-55K in adenovirus type 5 replication. J. Virol. 73, 5333–5344.

Harlow, E., Franza Jr., B.R., Schley, C., 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. 55, 533–546.

Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26, 365–369.

Howe, J.A., Bayley, S.T., 1992. Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. Virology 186, 15–24.

Howe, J.A., Mymryk, J.S., Egan, C., Branton, P.E., Bayley, S.T., 1990. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. U.S.A. 87, 5883–5887.

Howe, J.A., Demers, G.W., Johnson, D.E., Neugebauer, S.E., Perry, S.T., Vaillancourt, M.T., Faha, B., 2000. Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. Mol. Ther. 2, 485–495.

Hubberstey, A.V., Pavliv, M., Parks, R.J., 2002. Cancer therapy utilizing an adenoviral vector expressing only E1A. Cancer Gene Ther. 9, 321–329.

Huyghe, B.G., Liu, X., Sutjipto, S., Sugarman, B.J., Horn, M.T., Shepard, H.M., Scandella, C.J., Shabram, P., 1995. Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography. Hum. Gene Ther. 6, 1403–1416.

Imler, J.L., Chartier, C., Dreyer, D., Dieterle, A., Sainte-Marie, M., Faure, T., Pavirani, A., Mehtali, M., 1996. Novel complementation cell lines derived from human lung carcinoma A549 cells support the growth of E1-deleted adenovirus vectors. Gene Ther. 3, 75–84.

Jones, N., Shenk, T., 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. U.S.A. 76, 3665–3669.

Lee, W.P., Tai, D.I., Tsai, S.L., Yeh, C.T., Chao, Y., Lee, S.D., Hung, M.C., 2003. Adenovirus type 5 E1A sensitizes hepatocellular carcinoma cells to gemcitabine. Cancer Res. 63, 6229–6236.

Lochmuller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G., Acsadi, G., 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. Hum. Gene Ther. 5, 1485–1491.

Louis, N., Evelegh, C., Graham, F.L., 1997. Cloning and sequencing of the cellular–viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology 233, 423–429.

Lu, W., Lin, J., Chen, J., 2002. Expression of p14ARF overcomes tumor resistance to p53. Cancer Res. 62, 1305–1310.

Marcellus, R.C., Teodoro, J.G., Wu, T., Brough, D.E., Ketner, G., Shore, G.C., Branton, P.E., 1996. Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. J. Virol. 70, 6207–6215.

McKinnon, R.D., Bacchetti, S., Graham, F.L., 1982. Tn5 mutagenesis of the transforming genes of human adenovirus type 5. Gene 19, 33–42.

Meier, O., Greber, U.F., 2003. Adenovirus endocytosis. J. Gene Med. 5, 451–462.

Murakami, P., Pungor, E., Files, J., Do, L., van Rijnsoever, R., Vogels, R., Bout, A., McCaman, M., 2002. A single short stretch of homology between adenoviral vector and packaging cell line can give rise to cytopathic effect-inducing, helper-dependent E1-positive particles. Hum. Gene Ther. 13, 909–920.

Mymryk, J.S., Shire, K., Bayley, S.T., 1994. Induction of apoptosis by adenovirus type 5 E1A in rat cells requires a proliferation block. Oncogene 9, 1187–1193.

Nahle, Z., Polakoff, J., Davuluri, R.V., McCurrach, M.E., Jacobson, M.D., Narita, M., Zhang, M.Q., Lazebnik, Y., Bar-Sagi, D., Lowe, S.W., 2002. Direct coupling of the cell cycle and cell death machinery by E2F. Nat. Cell Biol. 4, 859–864.

Oh, T., Kim, C., Woo, S., Kim, T., Jeong, D., Kim, M., Lee, S., Cho, H., An, S., 2004. Development and clinical evaluation of a highly sensitive DNA microarray for detection and genotyping of human papillomaviruses. J. Clin. Microbiol. 427, 3272–3280.

Palu, G., Parolin, C., Takeuchi, Y., Pizzato, M., 2000. Progress with retroviral gene vectors. Rev. Med. Virol. 10, 185–202.

Querido, E., Marcellus, R.C., Lai, A., Charbonneau, R., Teodoro, J.G., Ketner, G., Branton, P.E., 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. 71, 3788–3798.

Ramachandra, M., Rahman, A., Zou, A., Vaillancourt, M., Howe, J.A., Antelman, D., Sugarman, B., Demers, G.W., Engler, H., Johnson, D., Shabram, P., 2001. Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy. Nat. Biotechnol. 19, 1035–1041.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., White, E., 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. U.S.A. 89, 7742–7746.

Sarnow, P., Ho, Y.S., Williams, J., Levine, A.J., 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell 28, 387–394.

Shabram, P.W., Giroux, D.D., Goudreau, A.M., Gregory, R.J., Horn, M.T., Huyghe, B.G., Liu, X., Nunnally, M.H., Sugarman, B.J., Sutjipto, S., 1997. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. Hum. Gene Ther. 8, 453–465.

Shenk, T., 1996. Adenoviridae: the viruses and their replication. In: K.D.M., Fields, B.N., Howley, P.M. (Eds.), Fields Virology, vol. 2. Lippincott-Raven, Philadelphia, PA.

Shisler, J., Duerksen-Hughes, P., Hermiston, T.M., Wold, W.S., Gooding, L.R., 1996. Induction of susceptibility to tumor necrosis factor by E1A is dependent on binding to either p300 or p105-Rb and induction of DNA synthesis. J. Virol. 70, 68–77.

Smith, C.D., Craft, D.W., Shiromoto, R.S., Yan, P.O., 1986. Alternative cell line for virus isolation. J. Clin. Microbiol. 24, 265–268.

Teodoro, J.G., Shore, G.C., Branton, P.E., 1995. Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. Oncogene 11, 467–474.

Ueno, N.T., Bartholomeusz, C., Herrmann, J.L., Estrov, Z., Shao, R., Andreeff, M., Price, J., Paul, R.W., Anklesaria, P., Yu, D., Hung, M.C., 2000. E1A-mediated paclitaxel sensitization in HER-2/neu-overexpressing ovarian cancer SKOV3.ip1 through apoptosis involving the caspase-3 pathway. Clin. Cancer Res. 6, 250–259.

White, E., Cipriani, R., Sabbatini, P., Denton, A., 1991. Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. 65, 2968–2978.

Whyte, P., Williamson, N.M., Harlow, E., 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell 56, 67–75.

Wills, K.N., Maneval, D.C., Menzel, P., Harris, M.P., Sutjipto, S., Vaillancourt, M.T., Huang, W.M., Johnson, D.E., Anderson, S.C., Wen, S.F., et al., 1994. Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. Hum. Gene Ther. 5, 1079–1088.

Winberg, G., Shenk, T., 1984. Dissection of overlapping functions within the adenovirus type 5 E1A gene. EMBO J. 3, 1907–1912.

Zhou, Z., Jia, S.F., Hung, M.C., Kleinerman, E.S., 2001. E1A sensitizes HER2/neu-overexpressing Ewing's sarcoma cells to topoisomerase II-targeting anticancer drugs. Cancer Res. 61, 3394–3398.

Zhu, J., Grace, M., Casale, J., Chang, A.T., Musco, M.L., Bordens, R., Greenberg, R., Schaefer, E., Indelicato, S.R., 1999. Characterization of replication-competent adenovirus isolates from large-scale production of a recombinant adenoviral vector. Hum. Gene Ther. 10, 113–121.